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(54) COMPOSITIONS FAVORISANT LA CHONDROGENESE
(54) COMPOSITIONS FOR PROMOTING CHONDROGENESIS

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(54) **COMPOSITIONS FAVORISANT LA CHONDROGENESE**
(54) **COMPOSITIONS FOR PROMOTING CHONDROGENESIS**

COMPOSITIONS FOR PROMOTING CHONDROGENESIS

Field of the Invention

The invention relates to compositions for promoting chondrogenesis.

Background of the Invention

Retinoic acid (RA) is known to have an important signalling role in the regulation of embryonic development and cell differentiation. The biological effects of RA are transduced via two classes of nuclear receptors, designated retinoic acid receptors (RARα, RARβ and RARγ) and retinoid X receptors (RXRα, RXRβ and RXRγ) (Giguere et al., (1987), Nature, 350, 624-629).

Many studies have been made of the role of RA and its receptors in the process of chondrogenesis and skeletal development. For example, addition of RA and RAR-specific agonists has been shown to inhibit cartilage formation in limb bud micromass cultures, and act as a teratogen *in vivo* to negatively affect skeletal development (Kistler, 1987; Kochhar, 1973; Kochhar and Aydelotte, 1974; Kwasigroch and Kochhar, 1980).

In contrast, addition of retinoic acid to micromass cultures stimulated cartilage formation (Ide and Aono, 1988; Paulsen et al., 1994a; Paulsen et al., 1994b).

Addition of an RAR antagonist completely reversed the inhibitory action of an RAR agonist on chondrocyte differentiation in rat and mouse embryo limb bud mesenchymal cells *in vitro* (Eckhardt et al., (1994), Toxicol. Letters, 70, 299-308; Kocchar et al., (1998), Int. J. Dev. Biol., 42, 601-608). These authors, however, found that the antagonist alone had no effect on limb bud mesenchymal cell differentiation.

It was unclear from these studies whether RARs functioned to inhibit chondrogenesis or to stimulate that process.

Summary of the Invention

In accordance with one embodiment, the present invention provides a pharmaceutical composition, comprising an effective amount of an RAR antagonist and, optionally, a pharmaceutically acceptable carrier.

In accordance with a further embodiment, the invention provides a method for stimulating cartilage formation in a vertebrate, the method comprising administering to the vertebrate an effective cartilage formation stimulating amount of an RAR antagonist.

In accordance with a further embodiment, the invention provides a method for treating a bone fracture in a subject, comprising administering to the subject an effective amount of an RAR antagonist.

In accordance with a further embodiment, the invention provides a method for enhancing osseous integration of orthopedic or dental implants in a subject comprising administering to the subject an effective amount of an RAR antagonist.

In accordance with a further embodiment, the invention provides a method for treating arthritis in a subject, comprising administering to the subject an effective amount of an RAR antagonist.

In accordance with a further embodiment, the invention provides a method for treating arthritis in a subject, comprising administering to the subject chondrogenic cells treated with an effective amount of an RAR antagonist.

Summary of the Drawings

Figure 1: Transgene-expressing Cells are Excluded from Cartilage Nodules.

(A-C) Wild-type fore limb cultures were stained with alcian blue on days 2. 4 and 6.

(D-H) Transgenic fore limb cultures stained with magental-gal followed by alcian blue at days 2, 4 and 6.

(D) Higher magnification of day 4 transgenic fore limb cultures. Transgene-expressing cells are excluded from the cartilage nodules.

(H) Day 4 transgenic hind limb cultures. Transgene-expressing cells are much more abundant compared to in fore limb cultures, and hence only a few small cartilage nodules have formed.

Bar: (A-C, E-H) 1.0 mm (D) 0.4 mm

Figure 2A: Transgene-expressing Cells Have a Prechondrogenic Phenotype.

(A-H) Whole mount in situ hybridization of wild-type and transgenic cultures was carried out using probes characteristic of specific stages of chondrogenesis.

(A) Col II expression is very abundant in wild-type cultures.

(B) Col II expression in transgenic cultures is much weaker and has a more widespread distribution.

(C, E, G) In wild-type cultures, N-cad, gli-1, and col I are all abundantly expressed in perinodular regions but are only weakly expressed in the core of the nodules.

(D, F, H) In transgenic cultures, N-cad, gli-1 and col-I are expressed throughout the cultures with no apparent downregulation as seen in the center of the nodules of wild-type cultures.

Bar: (A-H) 0.4 mm

Figure 2B: Northern blot analysis using total hind limb bud RNA indicates that col II is downregulated in transgenic hind limbs at E 14.5, and gli-1 is downregulated in transgenic hind limbs at E 12.5.

Figure 3: Transgene-expressing Cells Respond Differently to BMP-2.

(A) Quantitative analysis of nodule formation in vitro. There are fewer cartilage nodules in the fore and hind limb cultures of untreated transgenic cultures compared to those in untreated wild-type cultures. BMP-2 treatment increases the number of nodules in both wild-type and transgenic cultures, however, the increase seen in wild-type cultures is more dramatic.

(B-D) Wild-type cultures treated with BMP-2 were stained with alcian blue at days 2, 4 and 6. In response to BMP-2, there is a noticeable increase in nodule number and in nodule size, with cartilage nodules located at the periphery of the cultures becoming the largest.

(F-H) Transgenic cultures treated with BMP-2 were stained with magenta-gal followed by alcian blue at days 2, 4 and 6. In response to BMP-2 there is an increase in nodule number and in nodule size. There are also many more condensations of transgene-expressing cells.

(E) Higher magnification of day 4 wild-type cultures treated with BMP-2.

(I) Higher magnification of day 4 transgenic cultures treated with BMP-2. BMP-2 stimulates condensation of transgene-expressing cells, but they remain excluded from cartilage nodules.

Bar: (A-C, E-G) 1.0 mm (D, H) 0.4 mm

Figure 4: Col II Expression is Altered in Transgenic Cultures.

(A, B) Col II expression in wild-type and transgenic cultures respectively. In wild-type cultures, col II is abundantly expressed within cartilage nodules and is much weaker in transgenic cultures with a less distinct expression pattern.

(C) In response to BMP-2 the number of cartilage nodules in wild-type cultures that express col II abundantly increases.

(D) In transgenic cultures treated with BMP-2 there are a few nodules with abundant col II expression and several condensations with weaker expression.

Bar: (A-D) 0.4 mm

Figure 5: BMP-2 and AGN194301 Exhibit Different Chondrogenic Stimulatory Properties.

(A) Untreated wild-type cultures were stained with alcian blue on day 6.

(B, C) Wild-type cultures were treated with BMP-2 for the first two days and three days of culture, respectively and stained with alcian blue on day 6. These cultures resemble untreated cultures.

(D) Wild-type cultures were treated continuously with BMP-2 and stained with alcian blue on day 6.

(E, F) Wild-type cultures were treated after two or three days respectively and stained with alcian blue on day 6. These cultures resemble cultures that were treated continuously with BMP-2.

(G) Untreated wild-type cultures were stained with alcian blue on day 8.

(H, I) Wild-type cultures were treated with AGN194301 for the first two days and three days of culture, respectively, and stained with alcian blue on day 8. These cultures resemble cultures treated continuously with AGN194301 in that there are several more smaller nodules.

(J) Wild-type cultures were treated continuously with AGN194301 and stained with alcian blue on day 8.

(K, L) Wild-type cultures were treated after two or three days respectively and stained with alcian blue at day 8. The size and number of cartilage nodules is less compared to cultures treated continuously.

Bar: (A-L) 1.0 mm

(M) Quantification of Cartilage Nodule Formation in Response to BMP-2 and AGN194301. In response to BMP-2, or AGN194301 alone, there is a dramatic increase in the number of nodules that form. When BMP-2 and AGN194301 are added together, the number of nodules formed is greater than when each is added alone.

Figure 6: Loss of BMP signaling has an inhibitory effect on nodule formation that is rescued by AGN 194301.

(A) Wild-type cultures were stained with alcian blue on day 6.

(B) Wild-type cultures treated with Noggin were stained with alcian blue on day 6. Noggin-treated cultures show a dramatic decrease in nodule formation.

(C) Wild-type cultures treated with 1 uM AGN194301 were stained with alcian blue on day 6. The antagonist alone dramatically stimulates nodule formation.

(D) Wild-type cultures were treated with 10ng/ml Noggin and 1 mM AGN194301. When noggin and AGN 194301 are added together, there is no apparent decrease in nodule formation when compared to untreated cultures.

Bar: (A-D) 0.4 mm

Figure 7: Quantification of Cartilage Nodule Formation in Response to Noggin and AGN194301. Treatment of noggin alone dramatically reduces the number of cartilage nodules that form. When noggin and AGN194301 are both added to cultures the number of cartilage nodules is comparable to that in untreated cultures.

Detailed Description of the Invention

The present inventors have found that RAR antagonist compounds can act as potent stimulators of chondrogenesis or cartilage formation.

The RAR antagonist AGN 194301, shown in Table 1, has been shown to stimulate cartilage formation and promote chondroblast differentiation. AGN 194301 is a potent antagonist of RARα, with a high affinity for that receptor. It has a lower affinity for RARβ and RARγ, but does also act as an antagonist of these receptors.

In accordance with one embodiment of the invention, chondrogenesis-stimulating RAR antagonists comprise antagonist compounds which are highly effective against RARα and also antagonise RARβ and RARγ. Those of ordinary skill in the art are able to screen candidate compounds to identify compounds having such an RAR antagonist profile by methods available in the scientific literature, for example as described in Teng et al., (1997), J. Med. Chem., 40, 2445-2451.

In accordance with a preferred embodiment, chondrogenesis-stimulating RAR antagonists comprise mono- or di-fluoro substituted methylchromenes, such as AGN 194301. These compounds may be synthesised by conventional chemical synthetic methods. For example, AGN 194301 may be synthesised as described in Teng et al., (supra).

The chondrogenesis-stimulating RAR antagonists described herein are useful for the treatment and management of skeletal problems resulting from disease or trauma in vertebrates, including humans and other mammals, including horses. They may be used in any situation in which increased chondrogenesis is desired.

Therapeutic applications of these antagonists include:

(a) treatment of bone fractures, to stimulate new cartilage formation and accelerate bone repair through endochondral ossification.

A pharmaceutical composition comprising at least one chondrogenesis-stimulating RAR antagonist is applied locally to a fracture site, for example by means of a biodegradable sponge, gel or paste.

(b) treatment of orthopedic or dental implants to enhance or accelerate osseous integration. A pharmaceutical composition comprising at least one

chondrogenesis-stimulating RAR antagonist is applied locally to the site of desired osseous integration.

(c) treatment of arthritis, either osteoarthritis or other types of arthritis including rheumatoid arthritis:

(i) to reverse or slow cartilage degeneration, a pharmaceutical composition comprising at least one chondrogenesis-stimulating RAR antagonist would be applied locally through intra-articular injection or in combination with a viscosupplement. The composition could be provided in either a fast-release or slow-release formulation; or

(ii) for treatment of cells during osteochondral autograft or allograft transplantations (Minas et al., (1997), Orthopedics, 20, 525-538). In autograft transplantations, chondrogenic cells or cells with chondrogenic potential are removed from a patient (e.g. rib) and used to fill a cartilaginous lesion. An alternative method involves expanding these cells *in vitro*, then implanting them into a cartilaginous lesion. A pharmaceutical composition comprising at least one chondrogenesis-stimulating RAR antagonist would be used to treat the cells prior to engraftment and/or after engraftment through intra-articular injection.

The pharmaceutical compositions of the invention could also be used in combination with other chondrogenic stimulators, e.g. bone morphogenetic proteins (BMPs), to enhance and/or maintain the effects of these materials.

The RAR antagonist compositions described herein appear to have a more dramatic effect on chondrocyte function than the BMPs and show longer-lived effects. The RAR antagonists are also cheaper to manufacture than BMPs.

Those of ordinary skill in the art are familiar with various methods of formulating pharmaceutical compositions for local administration in diseases such as arthritis. For example, Adams et al., (1995), Osteoarthritis & Cartilage, 3, 213-225, describes viscosupplementation in osteoarthritis; Wozney et al.,

(1998), Clin. Ortho. Rel. Res., 346, 26-37, describes delivery methods used for BMPs to effect bone repair and formation.

Limb Bud Development

Patterning of the vertebrate limb bud relies on the cooperative action of several signaling centres. Signals have been identified that emanate from the apical ectodermal ridge (AER), ventral ectoderm (VE) and a region within the distal posterior margin termed the zone of polarizing activity (ZPA). The AER is important in specifying proximodistal axial identity, while the VE and ZPA are important in determining dorsal/ventral and anteroposterior identity, respectively. Each is essential for the proper patterning and outgrowth of the limb, and their perturbation results in defects of the paraxial skeleton. The mechanism by which these patterning cues influence mesenchyme commitment is thought to occur within the progress zone (PZ) which underlies the AER. Cells within the progress zone receive signals from all three signaling centres, integrating them into a positional identity that is fixed as the cells leave the PZ during limb outgrowth. Shortly thereafter this positional identity is translated into a cell identity and the corresponding differentiation program is initiated. With respect to skeletal formation, these signals culminate in the commitment of mesenchymal cells to the chondrocytic lineage.

Bones within the limb are formed from a cartilage precursor and the cartilage forms from condensed mesoderm. These condensations represent the earliest stages of limb patterning and are considered to be the forbears of the mature limb bones (Ede, 1983). Following condensation, the mesodermal cells in the interior of each condensation differentiate into chondrocytes. This differentiation occurs in concert with limb outgrowth, such that proximal mesenchymal cells (close to the body wall) that are fated to become chondrocytes differentiate prior to more distal cells. The spatiotemporal regulation of mesenchyme differentiation into chondrocytes is a crucial step in

endochondral bone formation in that it preserves the pattern of the bone primordia established earlier in limb development and provides a suitable matrix for subsequent ossification. Despite the importance of this stage in skeletal development, mechanisms that control mesenchyme differentiation into chondrocytes are poorly understood.

During limb outgrowth, signals that promote as well as inhibit chondrogenesis are important determinants in limb ontogeny (Wolpert, 1990). Many molecules have been identified that promote chondrogenesis *in vivo* and *in vitro*. Some of these include members of the transforming growth factor (TGF)-b superfamily, bone morphogenetic proteins (BMPs), *gdf-5*, and TGF-b -1, -2 and -3 (Hogan, 1996; Kingsley, 1994; Moses and Serra, 1996; Wozney and Rosen, 1998). The importance of *bmp-5* and *gdf-5* in limb development is illustrated by the recent mapping of the classical mouse mutations for short ear and brachypod to their respective loci (Kingsley et al., 1992; Storm et al., 1994). Both of these mutants exhibit malformations in appendicular skeletal formation which, for the case of brachypod, has been attributed to a defect in chondrogenesis (Duke and Elmer, 1977). Mice deficient in BMP-6 or BMP-7 also present with skeletal defects including delay in the ossification of the sternum in *bmp-6* -/- animals (Solloway et al., 1998) and polydactyly in the hind limbs of *bmp-7* -/- animals (Luo et al., 1995). Bmp-2 and -4 are expressed in condensing mesenchyme early in limb development then become localized to the interdigital mesenchyme and perichondrium (Jones et al., 1991; Lyons et al., 1990; Rosen et al., 1989). Mouse embryos devoid of BMP-2 or BMP-4 do not survive beyond E9.5 (Winnier et al., 1995; Zhang and Bradley, 1996) making it difficult to elucidate their exact roles in skeletal development. It is known, however, that exogenously added BMP-2 or -4 under certain circumstances leads to overgrowth of the limb cartilages (Duprez et al., 1996; Duprez et al., 1996). Loss or gain-of-function of noggin, an inhibitor of BMP-2, -4 and -7, contributes to either overgrowth or a reduction in limb cartilages,

respectively (Brunet et al., 1998; Capdevila and Johnson, 1998). Together, these observations suggest an important role for the BMPs, especially BMPs -2 and -4 in formation of the cartilaginous elements of the limb bud.

It is well established that retinoic acid (RA), the active derivative of vitamin A is essential for normal embryonic development. Exposure of embryos to excess RA results in a range of defects depending not only on the dose of RA, but also on the timing of its administration (Shenefelt, 1972). For instance when administered to E 11.5 to E 14.5 mouse embryos, large doses of RA cause limb defects (Kochhar, 1973; Kwasigroch and Kochhar, 1980). This period during which RA treatment has the most dramatic effects on limb formation coincides with the timing of chondrogenesis in the limb bud.

RA exerts most of its biological effects primarily through receptors belonging to the steroid hormone family of nuclear receptors. There are two subfamilies of nuclear retinoid receptors, the RA receptors (RARs) and the retinoid X receptors (RXRs), with three subtypes of each (a, b, and g) (Chambon, 1996; Mangelsdorf et al., 1994). All three subtypes of the RARs appear to have a fundamental role in chondrogenesis (for review see Underhill and Weston, 1998), and references therein). The expression patterns of the RARs in the developing limb are consistent with their proposed roles in skeletal formation. Between E9.5 and E11.5, RARa and g are expressed throughout the limb mesenchyme (Dolle et al., 1989; Ruberte et al., 1990). Beyond this stage, RARa is downregulated within the cartilaginous areas, while RARg expression remains localized to these areas. RARa expression becomes restricted to the interdigital region (IDR) overlapping with RARb expression at this time, and is also present in the perichondrium. In addition to being present in the IDR, RARb is expressed in the interior, anterior, and posterior necrotic zones (Dolle et al., 1989; Mendelsohn et al., 1991). While null mutants of either RARa, RARb or RARg exhibit no limb skeletal malformations (Ghyselinck et al., 1997; Lohnes et al., 1993; Lufkin et al., 1993; Luo et al., 1995), compound

homozygous null alleles of RARa and RARg exhibit a range of severe limb abnormalities from reductions to duplications (Lohnes et al., 1994). Thus, these results demonstrate an important function for the RARs in skeletal development.

To further explore RAR function in limb development we have used an existing transgenic line of mice that overexpress a weak constitutively active RARa1 (caRARa) in the developing limb bud (Cash et al., 1997). Transgenic animals present with a number of limb skeletal abnormalities as a result of transgene-mediated inhibition of cartilage formation. Here we report that transgene-expressing cells do not differentiate into chondroblasts, but instead remain as condensed mesenchyme. Conversely, an RARα antagonist was found to stimulate cartilage formation in wild-type limb mesenchymal cultures. Addition of BMP-2 or -4 to *in vitro* cultures of transgenic mesenchyme dramatically stimulates condensation of transgene-expressing mesenchymal cells but fails to induce chondroblast differentiation. More importantly, addition of this antagonist was found to rescue chondrogenesis in noggin treated wild-type cultures. Taken together, our results suggest that loss of RAR activity is necessary, and supersedes the requirement of BMPs, for chondroblast differentiation during limb outgrowth.

Regulation of chondroblast differentiation is critical for the proper formation of the appendicular skeleton . Herein we show that RAR activity has a fundamental role in controlling the transition of prechondrogenic cells to chondroblasts, and that the loss of RAR activity stimulates cartilage formation. The importance of RARs in mediating chondroblast differentiation is further exemplified by the observations that addition of BMP-2 and -4 is not sufficient to rescue RARα expressing prechondrogenic cells, while an RARα specific antagonist is able to rescue cartilage formation in noggin-treated cultures. Therefore, BMPs and RARs function in a sequential manner to orchestrate chondroblast differentiation during limb bud outgrowth.

Function of RARs during skeletal development

Overexpression of a caRARα in limb mesenchyme inhibits chondroblast differentiation and maintains the prechondrogenic cell phenotype. The phenotype of cells expressing the transgene is consistent with that of condensed mesenchymal cells and perichondrial cells. Both of these cell types are similar in that they are chondroprogenitors which that have not yet undergone chondroblast differentiation. Condensed mesenchymal cells and perichondrial cells both have continued expression of N-cadherin, gli-1, and col-I but express col II only weakly--a pattern of expression that is seen in transgenic cultures. The normal expression pattern of RARα in the developing limb is consistent with its absence being essential for the transition from a chondroprogenitor to a chondroblast. RARα is expressed in the prechondrogenic condensations, the perichondrium, and in the interdigital region, but is downregulated in newly formed cartilaginous elements. The importance of the loss of RARα signal is further demonstrated by experiments in which the RARα specific antagonist AGN194301 increased cartilage nodule formation in primary cultures by 60%. Hence, the proper regulation of RARα activity is essential for normal cartilage development.

Surprisingly, RARα null mutants display no overt skeletal malformations of the appendicular skeleton with the exception that a certain proportion (approx. 60% of animals surviving to 1-2 monthes of age) of RARα null mutants have webbed digits (Lohnes et al., 1993; Lufkin et al., 1993). However, in micromass cultures, attenuation of RARα2 expression with an anti-sense oligonucleotide to RARα2 stimulated cartilage formation *in vitro* (Jiang et al., 1995). This suggests that RARα alone is not essential for the maintenance of the prechondrogenic cell fate, but one of the RARs may be able to substitute for this function, the most likely candidate being RARγ. RARγ null mutants also have no obvious appendicular skeletal malformations. RARα/RARγ compound homozygous mutants, however, exhibit a number of

skeletal defects including reductions, duplications, and to a lesser extent, ectopic cartilage formation within the interdigital region (Lohnes et al., 1994). Ectopic cartilages are also observed at a number of additional sites in these animals including the meninges, peritoneum, diaphragm, and semi-lunar cusps of the heart (Lohnes et al., 1994; Mendelsohn et al., 1994). In addition, the phalangeal joints of these animals are malformed. While RARα/β and RARβ/γ double knockouts exhibit some cartilaginous deficiencies, they do not present with any appendicular skeletal defects. More importantly, the skeletal defects in the RARα/γ double knockouts can be rescued, for the most part, by the single allele of RARα2.

Results from knockout studies combined with the in vivo expression patterns of RARs suggest that both RARα and RARγ are important in regulating chondroblast differentiation in the limb. RARβ knockouts exhibit no skeletal abnormalities, and RARβ mRNA is absent in precartilaginous condensations during limb ontogeny (Ghyse..Mendelsohn). Thus, the combined activity of RARα and RARγ, but not RARβ is likely involved in regulating cartilage differentiation in the limb bud. In other areas of the developing embryo, chondrogenesis may also rely on the actions of specific combinations of RARs.

Sequential action of BMPs and RARs in skeletal development

The BMPs have been shown to be important in many aspects of endochondral bone formation including the commitment and differentiation of mesenchymal cells to the chondrocytic lineage. During limb outgrowth *bmp-2/-4* are expressed within the condensing mesenchyme, the perichondrium and the interdigital region (Jones et al., 1991; Lyons et al., 1990; Rosen et al., 1996). Unfortunately, null mutants have not been informative in sorting out the function of BMPs-2/-4 in these regions (Winnier et al., 1995; Zhang and Bradley, 1996). Additional studies, utilizing dominant-negative or

constitutively active BMP type II receptors *in vitro* and *in vivo* have demonstrated, however, that BMP signaling (most likely BMP-2 and 4) is a requisite step in cartilage formation (Zou et al., 1997). These observations have been complemented by experiments in which BMPs were overexpressed in the developing chich limb. It has been demonstrated that the BMPs can stimulate cartilage formation (Duprez et al., 1996; Duprez et al., 1996) and modify skeletal element patterning in addition to stimulating apoptosis within the interdigital region (Macias et al., 1997; Yokouchi et al., 1996). Furthermore, loss or gain of function studies with noggin, an inhibitor of BMP-2 and -4 with lower affinity for BMP-7, has shown that BMP-2 and-4 are important in skeletal development and that regulation of BMP signaling is required for delineation of the various skeletal elements (Brunet et al., 1998; Capdevila and Johnson, 1998). As described herein, exogenously added noggin inhibits cartilage formation in micromass cultures. BMP-2 has been shown to stimulate the commitment and differentiation of pluripotent mesenchymal cells to the chondrocytic lineage (Ahrens et al., 1993; Wang et al., 1993). Thus, BMPs, especially -2/-4, are important in early skeletal development, and appear to have properties consistent with a functional importance in commitment and differentiation of mesenchymal cells to chondrocytes.

Overexpression of a caRARα prevents prechondrogenic cell differentiation, even in the presence of BMP-2 or -4. Both BMP-2 and -4 enhance the expression of the prechondrogenic phenotype by stimulating the formation of numerous condensations as confirmed by magenta-gal staining and by in sity hybridization with probes for N-cadherin, gli-1, col-I and col-II. Nonetheless, neither BMP-2 nor BMP-4 was able to induce chondroblast differentiation of transgene-expressing cells. These results suggest that the regulation of RAR activity operates downstream of BMP-signaling in the chondroblast differentiation sequence. Additional evidence to suggest this comes from the observations that inhibition of cartilage formation by noggin

can be circumvented by treatment of cultures with an RAR antagonist. During paraxial development, RARα, RARγ, bmp-2 and -4 are co-expressed in the condensing mesenchyme and in the perichondrium. Hence, these results suggest that during limb outgrowth BMP-2 and -4 are important in stimulating prechondrogenic cell condensation, whereas a loss of RARα activity is important in allowing the differentiation of these cells. In this manner, the BMP and RA-signaling pathways may function sequentially in the commitment and differentiation of mesenchymal cells, respectively, during limb outgrowth.

RAR function in chondroblast differentiation: Conservation in other developmental processes

A similar hierarchy of BMP and RA signaling may be operating in other developmental processes. BMPs-2 and-4 and RARs are co-expressed in a number of different regions within the developing embryo. As mentioned previously, BMP-2 and -4 are co-expressed with RARα, β, and γ in the interdigital region. Application of BMPs to the interdigital region enhances interdigital apoptosis (Macias et al., 1997; Yokouchi et al., 1996), while overexpression of a dominant-negative BMPIIR inhibits interdigital apoptosis and webbed digits (Zou and Niswander, 1996; Zou et al., 1997). In addition, loss of RARα, or loss of β and γ together, leads to a cessation of interdigital apoptosis and webbed digits. When cultured prior to the initiation of apoptosis, explants of the interdigital region will form cartilage unless RA is present in which case they will undergo apoptosis (Lee et al., 1994). Similarly, addition of RA to *in vitro* cultured limbs stimulates regression of the interdigital regions (Lussier et al., 1993). Together, these observations suggest that the BMPs and RA-signaling pathways coordinate interdigital cell death, in addition to chondrogenesis.

During heart formation BMP-2 and -4 appear to be important in specifying ventricular cardiomyocytes whereas the RARs regulate the

differentiation of this population of cells. Loss of RAR activity through inactivation of RXRα, RXRβ, RARα causes precocious differentiation of ventricular myocytes(Kastner et al., 1997) . Hence, RARs are functioning in the heart to regulate progenitor cell differentiation as they do within the limb.

One of the functions of RARs in limb development is to regulate the differentiation of skeletal progenitor cells. In this manner, RAR activity may specify the size of progenitor cell populations, and/or influence cell fate decisions by modulating the competency of cells to respond to inductive signals, such as BMPs. The status of cellular RAR activity, therefore, appears to be an important determinant in the spatiotemporal regulation of cell differentiation in the developing limb, heart and spinal cord.

EXAMPLES

Methods

Preparation of micromass cultures

Micromass cultures were prepared from murine E11.25 to E11.75 fore and hind limb buds as previously described with the following modifications (Cash et al., 1997). After proteolytic digestion cells were filtered through a Cell Sieve (40uM, Falcon) to obtain a single cell suspension. Culture media (40% Dulbecco's modified Eagle's medium, 60% F12 was supplemented with fetal bovine serum to 10%, Gibco-BRL) was changed daily. BMP-2 or-4 (Genetics Institutes), AGN 194301 (Allergan Pharmaceuticals) and/or purified Xenopus noggin protein was added to culture media at a concentration of 10ng/ml, 1 uM and 10ng/ml, respectively. Addition/removal experiments included either adding or removing supplemented media on the indicated culture day, 24 hours after culture initiation was considered day 1. To detect transgene-expressing cells, cultures were fixed stained as previously described, with magenta-gal (BioSynth International Inc.) being substituted for X-gal. This was followed by alcian blue staining for cartilage-specific

glycosaminoglycans. Alcian blue staining of magenta-gal stained cultures turned the red precipitate to a purple color, this is a result of incubating magenta-gal stained cells at pH 1. This double-staining technique enables transgene-expressing cells to be localized with respect to alcian blue stained cartilage nodules.

Synthesis of Riboprobes

Riboprobes were synthesized in the presence of UTP-digoxigenin with the appropriate RNA polymerase and linearized template DNA according to the manufacturers directions (Boehringer Mannheim Corp., Indianapolis, IN). Riboprobe complementary to collagen type II gene, was generated from Bam H1 linearized pBluescript containing 1.1kb of the collagen type II gene containing the C-propeptide and transcribed *in vitro* with T7 RNA polymerase. Gli-1 riboprobe was transcribed from Not 1 linearized pBluescript containing a 1.6 kb fragment representing most of the zinc finger domain of gli-1. A 553 bp fragment of murine collagen type I (Phillips et al., 1992) was subcloned into pKS II (Stratagene), this was linearized with Xho1 and transcribed with T7 RNA polymerase. A Hind III (position 605) -BamH1 (position 1252) fragment from the mouse N-cadherin cDNA was subcloned into pKSII. This construct was linearized with Bam H1 and riboprobe synthesized with T7 RNA polymerase. For controls, sense riboprobes were synthesized from the aforementioned plasmids.

Whole-Mount *In situ* Hybridization of Micromass Cultures

In situ hybridizations were carried out on micromass cultures using a technique described previously (Cash et al., 1997), with minor modifications. After permeabilization using 10 μg/ml proteinase-K in phosphate buffered saline (PBS) supplemented with 0.05% Triton X-100, cells were post-fixed in

4% paraformaldehyde and 2% glutaraldehyde in PBS and hybridizations were carried out at 60° C instead of 55° C.

Transient Transfection Analysis

The ability of AGN194301 to inhibit all-trans RA induction of an RARE containing luciferase construct was performed in P19 embryonal carcinoma cells as previously described with some modification (Underhill et al., 1994). P19 cells were seeded at a density of 1.5 X 10^4 cells/well in 6 well plates. Cells were transfected using the calcium phosphate precipitation method with each well receiving 3.9 ug DNA (1.25 ug pW1RAREtk-lucif, 0.33ug pW1ActRARα/β/γ, .67 ug pW1Actβ-galactosidase and 1.65ug pGEM9zf(-)). Following transfection cells were washed and fresh media was added that contained 1 X 10^{-7} M all-trans RA and various amounts of AGN194301. Twenty-four hours later cell extracts were prepared, and luciferase and β-galactosidase activity measured. Luciferase activity was normalized with β-galactosidase activity to control for differences in transfection efficiency.

Northern Blot Analysis

Total RNA was isolated dissected and pooled limb buds from wild-type and transgenic embryos at various gestational stages with TriPure Isolation Reagent (Boerhinger Manheim). RNA samples were separated by electrophoresis of 15 μg aliquots in a 1% agarose-formaldehyde gel. RNA was then transferred to a Hybond-N nylon membrane (Amersham Life Science) and cross-linked by UV irradiation. Blots were pre-hybridized in Church's Buffer (7% SDS, 0.5 M NaPi pH 7.2, 1mM EDTA, 1% BSA) at 65° C for at least 30 min. Radiolabeled DNA probes were synthesized by random priming (ref.) with the appropriate cDNA insert fragments . Hybridizations were carried out overnight at 60° C. Following hybridization blots were washed with wash

buffer (250mM NaPi, 10% SDS) three times for 15 min. at 65°C, and exposed to BioMax X-ray film at -80°C for 1-4 days.

Example 1: Transgene-expressing cells do not contribute to cartilage nodule formation

To further understand the role of the RARs, specifically RARα, during cartilage formation, we used a previously described transgenic mouse model (Cash et al., 1997). Overexpression of a weak constitutively active RARα during limb development leads to various congenital malformations of the limb that are reminiscent of those observed in RA teratogenicity. During chondroblast differentiation, RARα expression is down-regulated. The continued expression of RARα inhibits chondroblast differentiation leading to a cessation of cartilage formation and to skeletal deficiencies as observed in the transgenic mice. Limb mesenchyme from embryonic age (E) 11.5 transgenic embryos was used to set up micromass cultures to examine the cell fate of transgene-expressing cells during *in vitro* chondrogenesis. Shown in figure 1 is a time-course of cartilage nodule formation (day-2, day-4 and day-6) in wild-type (figure 1A, B, and C) and transgenic fore limb cultures (figure 1E, 1F, 1G) and hind limb cultures (figure 1H, day-4 shown). Consistent with previous observations there are many fewer alcian blue-stained nodules in the transgenic derived cultures than in the wild-type cultures. Using a combination of magenta-gal and alcian blue staining, we clearly show that transgene-expressing cells are, for the most part, excluded from the cartilage nodules (figure 1D) but appear to form condensations. Transgene-expressing cells fail to differentiate into chondroblasts in contrast to both non-transgene-expressing cells within the same cultures, and cells within wild-type cultures.

Example 2: Transgene-expressing cells have a prechondrogenic phenotype

Cartilage formation involves two well described steps: 1) condensation of mesenchymal cells; 2) differentiation of condensed mesenchyme to matrix-producing chondrocytes (Hall and Miyake, 1992). These two stages can be distinguished based on histological staining, however, using molecular markers is generally a more reliable method for determining the phenotype of cells at either stage. Previous studies have shown that condensed prechondrogenic cells express col II weakly and express N-cad, col I and gli-1 abundantly (Hall and Miyake, 1995; Marigo et al., 1996; Oberlender and Tuan, 1994). Upon differentiation of these cells, col II expression becomes much stronger, whereas expression of N-cad, col I, and gli-1 are downregulated. To further characterize the phenotype of the transgene-expressing cells, whole mount *in situ* hybridization was carried out to examine the expression patterns of col II, N-cad, col I and gli-1 in transgenic and wild-type cultures. In wild-type cultures, col II expression was very strong and localized primarily to the core of the cartilage nodules with weaker expression in regions surrounding the nodules (figure 2A). Expression of N-cad, col I and gli-1 in wild-type cultures was restricted to perinodular regions in condensed mesenchyme and was weakly expressed in the centre of the nodules (figure 2C, E and G). The expression patterns of these genes in the wild-type cultures is consistent with their expression *in vivo*. In transgenic cultures, however, their expression patterns would indicate that while condensations are present the condensed cells have not yet differentiated. There was no downregulation of N-cad, col I or gli-1, instead they were expressed throughout the condensations (figure 2D, F and H), whereas col II was only weakly expressed (figure 2B), resembling a pattern one would expect to see in condensations but not in cartilage nodules. These observations suggest that the transgene-expressing cells condense but do not undergo chondroblast differentiation.

Comparison of the distribution of col II expression in transgenic and wild-type cultures suggests that the transgenic cultures form a similar number of condensations as observed in wild-type cultures (figure 2A, B). Furthermore, the col II stained regions in transgenic and wild-type cultures are similar in size indicating that transgene-mediated inhibition of chondroblast differentiation was not a consequence of insufficient numbers of prechondrogenic cells (Hall and Miyake, 1992). Moreover, during the culture period the transgene-expressing cell aggregates continue to expand in size and staining intensity (figure 1E, 1F and 1G). Northern blot analysis has confirmed differential expression of col II and gli-1 between the hind limbs of wild-type and transgenic mice. By E 14.5, col II is downregulated in transgenic hind limbs (Figure 2I), whereas gli-1 is downregulated in transgenic hind limb at E 12.5 (Figure 2I). The *in situ* hybridization results combined with results from Northern blot analysis strongly suggest that the phenotype displayed by transgene-expressing cells is consistent with condensed prechondrogenic cells.

Example 3: Transgene-expressing cells are refractile to BMP-stimulated chondroblast differentiation

To further investigate the mechanism of transgene-mediated inhibition of chondroblast differentiation, we examined the expression of genes known to be important in chondrogenesis, namely the BMPs. Based on their expression in limb development and their well defined chondrogenic stimulatory properties we have focused our analysis on *bmps-2* and *-4*. Hence, one plausible explanation for the transgenic phenotype was either the reduced expression of *bmps* or overexpression of *noggin* which encodes an inhibitor of BMP signaling. Northern analysis using mRNA from hind limbs of transgenic and wild-type animals at E 11.5, 12.5, and E 14.5 demonstrated that neither *bmp2* nor *noggin* is differentially expressed in transgenic animals in comparison to wild-type animals (data not shown). The defect in transgenic limb

mesenchyme therefore is not likely a result of changes in expression of either of these two transcripts. However, these results do not preclude the possibility that transgene-expressing mesenchymal cells are unresponsive to BMPs.

To evaluate whether transgene-expressing cells are able to respond to BMPs, we treated transgenic and wild-type micromass cultures with 10 ng/ml BMP-2 and -4. Similar to previous reports, we demonstrated that addition of BMP-2 (figure 3A) and -4 (data not shown) dramatically increases the number of cartilage nodules in wild-type and transgenic cultures. BMP-2 treatment for six days increased the number of cartilage nodules by ~125% and ~115% in wild-type and transgenic fore limb cultures, respectively (figure 3A-I). Hence, addition of BMP-2 or –4 appears to rescue the chondrogenic defect present in transgenic cultures. To confirm this, BMP-2 treated cultures were stained with magenta-gal followed by alcian blue (figure 3F, G and H). As observed in non-treated transgenic cultures, few if any transgene-expressing cells were found to be present within the alcian blue stained cartilage nodules (figure 3I). Addition of BMP-2, however, stimulated condensation of transgene-expressing cells (figure 3F-I). This was confirmed by *in situ* hybridization, BMP-2 addition stimulated the formation of col II expressing nodules and weak col II condensations in wild-type and transgenic cultures, respectively (Figure 4). Condensations were observed as early as 2 days in culture and were still evident after 6 days in culture. Thus, although BMP-2 or -4 induces trangene-expressing cells to form precartilaginous condensations they are not sufficient to overcome transgene-mediated inhibition of prechondrogenic cell differentiation.

Example 4: Loss of retinoic acid receptor activity stimulates chondrogenesis

We have clearly demonstrated that the continued expression of RARα inhibits the transition of prechondrogenic cells to chondroblasts. Consequently,

we would expect that the abrogation of RARα activity would stimulate chondroblast differentiation and/or cartilage formation. RAR activity as used herein refers to the level of RA-induced transcriptional activity of the RARs. To examine the possibility that inhibition of RARα activity stimulates cartilage formation, we treated micromass cultures with the RARα-specific antagonist AGN194301. Addition of 1μM AGN194301 to wild-type micromass cultures lead to a dramatic increase in nodule number with no apparent increase in nodule size as compared to untreated control cultures (figure 5G, J, M). After 8 days in culture there were 60% more nodules in antagonist treated cultures than in untreated cultures (figure 5M). Similar to the inhibition of cartilage nodule formation observed in response to all-trans RA treatment of micromass cultures, addition of the RARα-specific agonist AGN 193836 at 1 uM, decreased the number of cartilage nodules that formed by 35% with the nodules staining only weakly with alcian blue. To confirm that the concentrations utilized in these experiments inhibited RARα activity specifically, we measured the level of RARα, RARβ and RARγ mediated RARE activation with 100nM all-trans RA in the presence of various concentrations of antagonist. At 1 uM of AGN194301, RARα signaling was inhibited to ~0.3% of controls, while RARβ and RARγ were inhibited to ~18% and ~26% of controls, respectively. Hence, most of the chondrogenic stimulatory properties of the antagonist appear to be mediated through inhibition of RARα, however, it cannot be entirely discounted that diminution of RARβ or RARγ signaling may have contributed to these results. Nonetheless, loss of RAR activity stimulates cartilage formation while increased RAR activity inhibits cartilage formation.

Example 5: The RAR antagonist and BMP-2 have different chondrogenic stimulatory properties

The loss of RAR activity and addition of BMP-2 both stimulate cartilage formation. In transgenic cultures, BMP-2 stimulated condensation but not differentiation of transgene-expressing cells, whereas a loss of RARα activity induced cartilage formation. These results suggest that the two factars: a) BMP-2 availability and b) RARα activity are important at different stages during chondrogenesis. To further delineate the role of BMP-2 and RARα in cartilage formation, we have used an approach that involves incubation of micromass cultures with AGN194301 or BMP-2 for different periods of time during culturing. To accomplish this BMP-2 or AGN194301 were added to cultures for the first 2 or 3 days then removed, or were added after 2 or 3 days of culturing. As has been shown previously (Roark and Greer, 1994), addition of BMP-2 at later culture periods yielded results that are comparable to experiments in which BMP-2 is added continuously from the start of culture (figure 5D-F). Conversely, addition of BMP-2 for the first 2 to 3 days of culture caused an increase in nodule number but this increase was not as dramatic as that observed upon adding BMP-2 later (ie: after 2 or 3 days). The average size of the nodules was also noticeably increased in cultures that were either continuously exposed to BMP-2 or treated after 2 or 3 days of culture (figure 5B-F). These large nodules radiate towards the outside of the culture and are, in part, a consequence of the recruitment of proliferating uncommitted cells present in the periphery of the culture into the nodules. Similar observations have been made with BMP-2 addition *in vivo* as Duprez *et al.* (Duprez et al., 1996) showed that expansion of skeletal elements in the presence of BMP-2 or -4 was at the expense of other cell populations in the developing chick limb bud. Hence, the consequences of BMP-2 addition are much more pronounced in cultures treated at later stages of culture and likely

reflect the ability of BMP-2 to stimulate commitment of mesenchymal cells to the chondrocytic lineage with subsequent recruitment into nodules.

In contrast to BMP-2 and -4, AGN194301 had its most pronounced stimulatory effect on nodule formation when it was present early in the culture period. When 1 μM AGN194301 was added to cultures for only the first 2 or 3 days, then removed, more cartilage nodules were formed compared to untreated cultures (figure 5G-I) or to cultures exposed for longer periods of time but untreated for the first 2 or 3 days (figure 5K, L). Interestingly, the increase in nodule number caused by early treatment of the antagonist was maintained for 4 or more days after its removal and was comparable to the increase in nodule number observed with continuous treatment (figure 5H-J). AGN194301, therefore, caused an increase in nodule formation, however the nodules were much smaller compared to cultures receiving antagonist at later culture times. When BMP-2 and AGN194301 were added together to micromass cultures at a concentration of 10ng/ml and 1 uM, respectively there was a ~100% increase in nodule number compared to untreated controls (figure 5M). BMP-2 addition alone to cultures initiated from the same limb buds caused a ~45% increase, whereas AGN194301 treatment caused an increase of ~60% (figure 5M). Together, these results suggest that the loss of RAR activity and the presence of a BMP signal are two events that act at different stages of the chondrogenic sequence as they exhibit markedly different chondrogenic stimulatory properties.

Example 6: Cartilage formation can be rescued in the absence of BMP-2/-4 signaling by addition of an RAR antagonist

Earlier experiments in which BMP-2 was unable to stimulate transgene-expressing cells to differentiate into chondroblasts suggested that loss of RARα activity regulates chondroblast differentiation downstream of a BMP-2/-4

mediated signal. If this were the case, loss of RARα would be expected to induce nodule formation even in the absence of BMP-2 and -4 signaling. To test this we treated wild-type micromass cultures with noggin, a soluble inhibitor of BMP-2, and -4. Addition of noggin to micromass cultures dramatically reduced cartilage nodule formation by ~84% in comparison to untreated controls (Fig 6A and B and Figure 7). This is consistent with results of overexpression of noggin in chick limb buds which resulted in the severely impaired development of cartilages (Capdevila and Johnson, 1998). Addition of 10 or 20 nM all-trans RA further diminished nodule formation in noggin-treated cultures (data not shown). In contrast, the addition of 1 uM AGN 194301 to noggin-treated cultures, stimulated cartilage nodule formation with there being little difference in the number of nodules that formed compared to untreated controls (figure 6A and C and Figure 7), albeit some of these nodules stained more weakly with alcian blue; as has been observed in AGN194301 treated-cultures. These results indicate that AGN 194301 is sufficient to rescue the effect of a loss of BMP signaling. These results, combined with studies in which BMP treatment of cells overexpressing RARα fails to stimulate chondroblast differentiation, strongly suggest that loss of RAR activity is important downstream of BMP signaling during chondrogenesis.

References

Adams, M. E., Atkinson, M. H., Lussier, A. J., Schulz, J. I., Siminovitch, K. A., Wade, J. P., and Zummer, M. (1995). The role of viscosupplementation with hylan G-F 20 (Synvisc) in the treatment of osteoarthritis of the knee: a Canadian multicenter trial comparing hylan G-F 20 alone, hylan G-F 20 with non-steroidal anti-inflammatory drugs (NSAIDs) and NSAIDs alone. Osteoarthritis & Cartilage 3, 213-25.

Ahrens, M., Ankenbauer, T., Schroder, D., Hollnagel, A., Mayer, H., and Gross, G. (1993). Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into distinct mesenchymal cell lineages. DNA Cell Biol. 12, 871-880.

Amos, B., and Lotan, R. (1990). Retinoid-sensitive cells and cell lines. Meth. Enz. 190, 217-225.

Brunet, L. J., McMahon, J. A., McMahon, A. P., and Harland, R. M. (1998). Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton [see comments]. Science 280, 1455-7.

Capdevila, J., and Johnson, R. L. (1998). Endogenous and ectopic expression of noggin suggests a conserved mechanism for regulation of BMP function during limb and somite patterning. Dev Biol 197, 205-17.

Cash, D. E., Bock, C., Schughart, K., Linney, E., and Underhill, T. M. (1997). Retinoic acid receptor alpha function in vertebrate limb skeletogenesis: a modulator of chondrogenesis. J. Cell Biol. 136, 445-457.

Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. FASEB J. 10, 940-954.

Dolle, P., Ruberte, E., Kastner, P., Petkovich, M., Stoner, C. M., Gudas, L. J., and Chambon, P. (1989). Differential expression of genes encoding alpha, beta and gamma retinoic acid receptors and CRABP in the developing limbs of the mouse. Nature 342, 702-705.

Duke, J., and Elmer, W. A. (1977). Effect of the brachypod mutation on cell adhesion and chondrogenesis in aggregates of mouse limb mesenchyme. J. Embryol. Exp. Morph. 42, 209-217.

Duprez, D., de H. Bell, E. J., Richardson, M. K., Archer, C. W., Wolpert, L., Brickell, P. M., and Francis-West, P. H. (1996). Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. Mechanisms of Development 57, 145-157.

Duprez, D. M., Coltey, M., Amthor, H., Brickell, P. M., and Tickle, C. (1996). Bone Morphogenetic Protein-2 (BMP-2) inhibits muscle development and promotes cartilage formation in chick limb bud cultures. Developmental Biology 174, 448-452.

Eckhardt, K., and Schmitt, G. (1994). A retinoic receptor alpha antagonist counteracts retinoid teratogenicity in vitro and reduced incidence and/or severity of malformations in vivo. Toxicol. Lett. 70, 299-308

Ede, D. (1983). Cellular condensations and chondrogenesis. In Cartilage: Development, Differentiation and Growth, B. K. Hall, ed. (New York: Academic Press), pp. 143-185.

Ghyselinck, N. B., Dupe, V., Dierich, A., Messaddeq, N., Garnier, J. M., Rochetteegly, C., Chambon, P., and Mark, M. (1997). Role of the Retinoic Acid Receptor Beta (RAR-Beta) During Mouse Development. Int. J. Dev. Biol. 41, 425-447.

Hall, B. K., and Miyake, T. (1995). Divide, accumulate, differentiate: cell condensation in skeletal development revisited. Int. J. Dev. Biol. 39, 881-893.

Hall, B. K., and Miyake, T. (1992). The membranous skeleton: The role of cell condensations in vertebrate skeletogenesis. Anat. Embryol. 186, 107-124.

Hogan, B. L. (1996). Bone morphogenetic proteins in development. Current opinion in genetics and development 6, 432-438.

Jones, C. M., Lyons, K. M., and Hogan, B. L. M. (1991). Involvement of Bone Morphogenetic Protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. Development 111, 531-542.

Kastner, P., Messaddeq, N., Mark, M., Wendling, O., Grondona, J. M., Ward, S., Ghyselinck, N., and Chambon, P. (1997). Vitamin A deficiency and mutations of RXRalpha, RXRbeta and RARalpha lead to early differentiation of embryonic ventricular cardiomyocytes. Development 124, 4749-58.

Kingsley, D. M. (1994). The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes & Dev. 8, 133-146.

Kingsley, D. M., Bland, A. E., Grubber, J. M., Marker, P. C., Russell, L. B., Copeland, N. G., and Jenkins, N. A. (1992). The mouse short ear skeletal

morphogenesis locus is associated with defects in a bone morphogenetic member of the TGF-beta superfamily. Cell 71, 399-410.

Kistler, A. (1987). Limb bud cell cultures for estimating the teratogenic potential of compounds. Arch. Toxicol. 60, 403-414.

Kochhar, D. M. (1973). Limb development in mouse embyros. I. Analysis of teratogenic effects of retinoic acid. Teratology 7, 289-295.

Kochhar, D. M., and Aydelotte, M. B. (1974). Susceptible stages and abnormal morphogenesis in the developing mouse limb, analysed in organ culture after transplacental exposure to vitamin A (retinoic acid). J. Embryol. Exp. Morph. 31, 721-734.

Kochhar, D. M., Jiang, H., Penner, J. D., Johnson, A. T., and Chandraratna, R. A. S. (1998). The use of a retinoid receptor antagonist in a new model to study vitamin a-dependent developmental events. Int. J. Dev. Biol. 42, 601-608.

Kwasigroch, T. E., and Kochhar, D. M. (1980). Production of congenital limb defects with retinoic acid: phenomenological evidence of progressive differentiation during limb morphogenesis. Anat. Embryol. 161, 105-113.

Lee, K. H., Li, F. C. H., Yung, W. T., Kung, J. L. S., Ng, J. N., and Cheah, K. S. E. (1994). Influence of digits, ectoderm, and retinoic acid on chondrogenesis by mouse interdigital mesoderm in culture. Dev. Dynam. 201, 297-309.

Lohnes, D., Kastner, P., Dierich, A., Mark, M., LeMeur, M., and Chambon, P. (1993). Function of retinoic acid receptor gamma in the mouse. Cell 73, 643-658.

Lohnes, D., Mark, M., Mendelsohn, C., Dolle, P., Dierich, A., Gorry, P., Gansmuller, A., and Chambon, P. (1994). Function of the retinoic acid receptors (RARs) during development (I) Craniofacial and skeletal abnormalities in RAR double mutants. Development 120, 2723-2748.

Lufkin, T., Lohnes, D., Mark, M., Dierich, A., Gorry, P., Gaub, M.-P., LeMeur, M., and Chambon, P. (1993). High postnatal lethality and testis degeneration in retinoic acid receptor alpha mutant mice. Proc. Natl. Acad. Sci. USA 90, 7225-7229.

Luo, G., Hofmann, C., Bronckers, A. L. J. J., Sohocki, M., Bradley, A., and Karsenty, G. (1995). BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. Genes & Dev. 9, 2808-2820.

Luo, J., Pasceri, P., Conlon, R. A., Rossant, J., and Giguere, V. (1995). Mice lacking all isoforms of retinoic acid receptor beta develop normally and are susceptible to the teratogenic effects of retinoic acid. Mech. Dev. 53, 61-71.

Lussier, M., Canoun, C., Ma, C., Sank, A., and Shuler, C. (1993). Interdigital soft tissue separation induced by retinoic acid in mouse limbs cultured in vitro. Int. J. Dev. Biol. 37, 555-564.

Lyons, K. M., Pelton, R. W., and Hogan, B. L. M. (1990). Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for Bone Morphogenetic Protein-2A (BMP-2A). Development 109, 833-844.

Macias, D., Ganan, Y., Sampath, T. K., Piedra, M. E., Ros, M. a., and Hurle, J. M. (1997). Role of BMP-2 and OP-1 (BMP-7) in programmed cell death and skeletogenesis during chick limb development. Development 124, 1109-1117.

Mangelsdorf, D. J., Umesono, K., and Evans, R. M. (1994). The retinoid receptors. In The Retinoids: Biology, Chemistry, and Medicine, M. B. Sporn, A. B. Roberts and D. S. Goodman, eds. (New York: Raven Press Ltd.), pp. 319-349.

Marigo, V., Johnson, R. L., Vortkamp, A., and Tabin, C. J. (1996). Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development. Dev. Biol. 180, 273-283.

McBurney, M. W., Jones-Villeneuve, E. M. V., Edwards, M. K. S., and Anderson, P. J. (1982). Control of muscle and neuronal differentiation in cultured embryonal carcinoma cell line. Nature 299, 165-167.

Mendelsohn, C., Ruberte, E., LeMeur, M., Morriss-Kay, G., and Chambon, P. (1991). Developmental analysis of the retinoic acid-inducible RAR-beta2 promoter in transgenic animals. Development 113, 723-734.

Minas, T., and Nehrer, S. (1997). Current concepts in the treatment of articular cartilage defects. Orthopedics 20, 525-538.

Moses, H. L., and Serra, R. (1996). Regulation of differentiation by TGF-beta. Current opinion in genetics and development 6, 581-586.

Oberlender, S. A., and Tuan, R. S. (1994). Spatiotemporal profile of N-cadherin expression in the developing limb mesenchyme. Cell Adhes Commun 2, 521-37.

Paulsen, D. F., Chen, W.-D., Okello, D., and Johnson, B. (1994a). Stage- and region-dependent responses of chick wing bud mesenchymal cells to retinoic acid in serum-free microcultures. Dev. Dynam. 201, 310-323.

Paulsen, D. F., Solursh, M., Langille, R. M., Pang, L., and Chen, W.-D. (1994). Stable, postion-related responses to retinoic acid by chick limb-bud mesenchymal cells in serum-free cultures. In Vitro Cell. Dev. Biol. 30A, 181-186.

Phillips, C. L., Morgan, A. L., Lever, L. W., and Wenstrup, R. J. (1992). Sequence analysis of a full-length cDNA for the murine Proa2(I) collagen chain: Comparison of the derived primary structure with human Proa2(I) collagen. Genomics 13, 1345-1346.

Roark, E. F., and Greer, K. (1994). Transforming growth factor-b and bone morphogenetic protein-2 act by distinct mechanisms to promote chick limb cartilage differentiation in vitro. Dev. Dynam. 200, 103-116.

Rosen, V., Thies, R. S., and Lyons, K. (1996). Signaling pathways in skeletal formation: A role for BMP receptors. Annals of the New York Academy of Science 785, 59-69.

Rosen, V., Wozney, J. M., Wang, E. A., Cordes, P., Celeste, A., McQuaid, D., and Kurtzberg, L. (1989). Purification and molecular cloning of a novel group of BMPs and localization of BMP mRNA in developing bone. Connect Tissue Res 20, 313-9.

Ruberte, E., Dolle, P., Krust, A., Zelent, A., Morriss-Kay, G., and Chambon, P. (1990). Specific spatial and temporal distribution of retinoic acid receptor gamma transcripts during mouse embryogenesis. Development 108, 213-222.

Shenefelt, R. E. (1972). Morphogenesis of malformations in hamsters caused by retinoic acid: relation to dose and stage at treatment. Teratology 5, 103-118.

Solloway, M. J., Dudley, A. T., Bikoff, E. K., Lyons, K. M., Hogan, B. L., and Robertson, E. J. (1998). Mice lacking Bmp6 function. Dev Genet 22, 321-39.

Storm, E. E., Huynh, T. V., Copeland, N. G., Jenkins, N. A., Kingsley, D. M., and Lee, S.-J. (1994). Limb alterations in brachypodism mice due to mutations in a new member of the TGFb-superfamily. Nature 368, 639-643.

Teng, M., Duong, T. T., Johnson, A. T., Klein, E. S., Wang, L., Khalifa, B., and Chandraratna, R. A. S. (1997). Identification of highly potent retinoic acid receptor alpha-selective antagonists. J. Med. Chem. 40, 2445-2451.

Underhill, T. M., Cash, D. E., and Linney, E. (1994). Constitutively active retinoid receptors exhibit interfamily and intrafamily promoter specificity. Mol. Endo. 8, 274-285.

Underhill, T. M., and Weston, A. D. (1998). Retinoids and their receptors in skeletal development. Micro. Res. Tech. 43, 137-155

Wang, E. A., Isreal, D. I., Kelly, S., and Luxenberg, D. P. (1993). Bone morphogenetic protein-2 causes commitment and diferentiation in C3H10T1/2 and 3T3 cells. Growth factors 9, 57-71.

Winnier, G., Blessing, M., Labosky, P. A., and Hogan, B. L. M. (1995). Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes & Dev. 9, 2105-2116.

Wolpert, L. (1990). Signals in limb development: STOP, GO, STAY and POSITION. J. Cell Sci. Suppl. 13, 199-208.

Wozney, J. M., and Rosen, V. (1998). Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair. Clin. Ortho. Rel. Res. 346, 26-37.

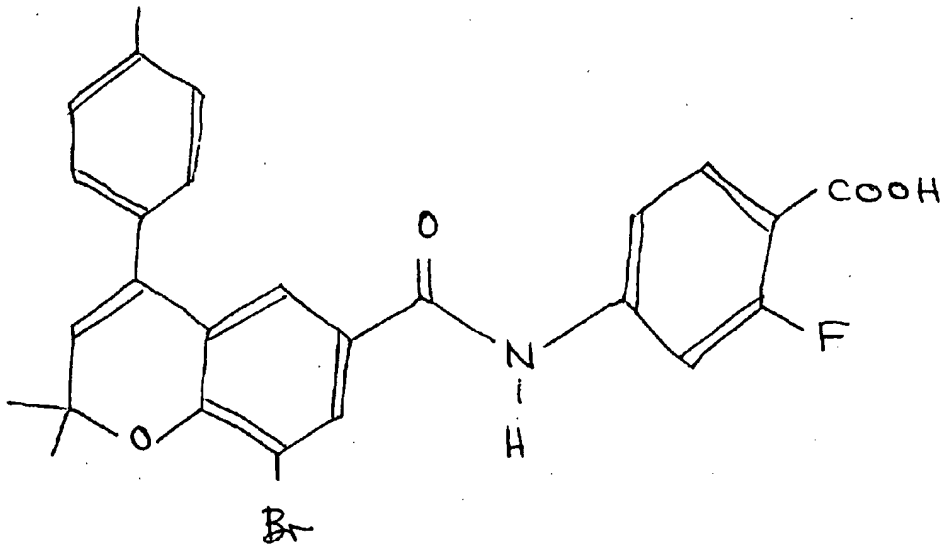
Yokouchi, Y., Sakiyama, J., Kameda, T., Iba, H., Suzuki, A., Ueno, N., and Kuroiwa, A. (1996). BMP-2/-4 mediate programmed cell death in chicken limb buds. Development 122, 3725-3734.

Zhang, H., and Bradley, A. (1996). Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development 122, 2977-2986.

Zou, H., and Niswander, L. (1996). Requirement for BMP signalling in interdigital apoptosis and scale formation. Science 272, 738-741.

Zou, H., Wieser, R., Massague, J., and Niswander, L. (1997). Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. Genes & Dev. 11, 2191-2203.

TABLE 1



AgN 194301

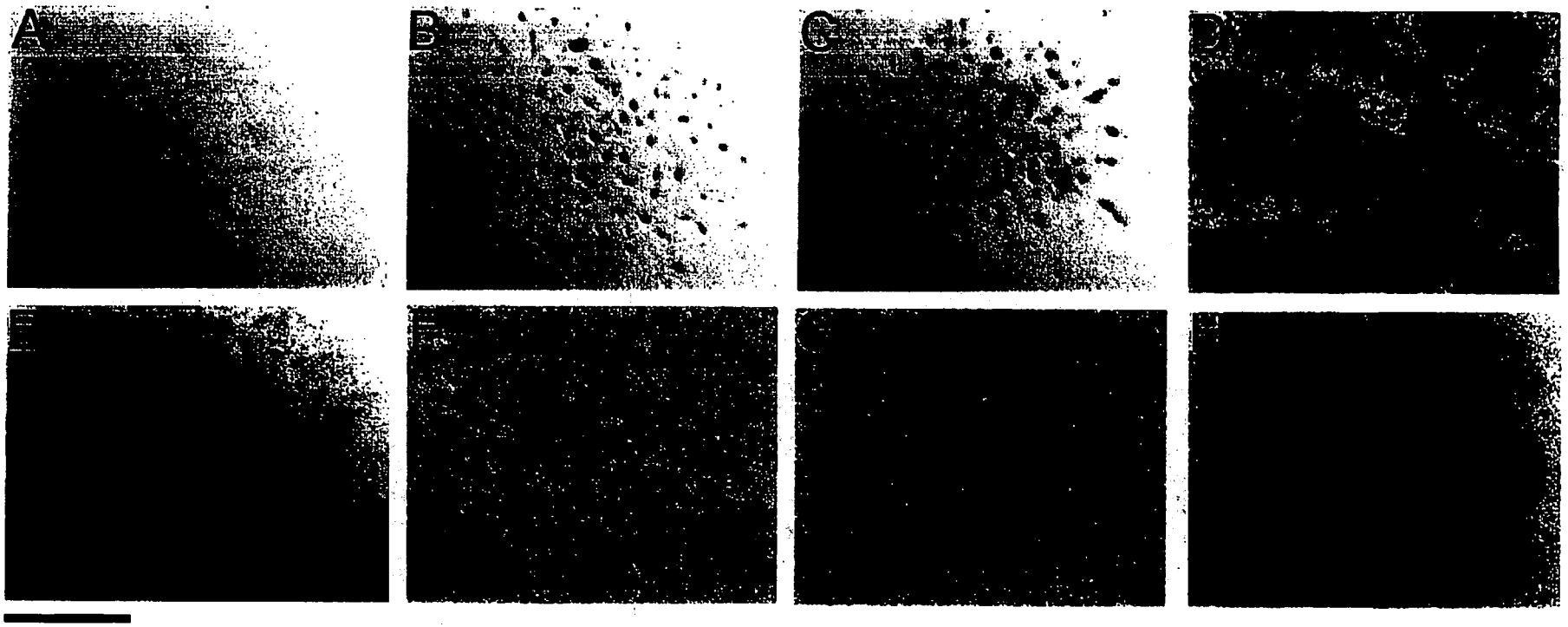


FIGURE 1

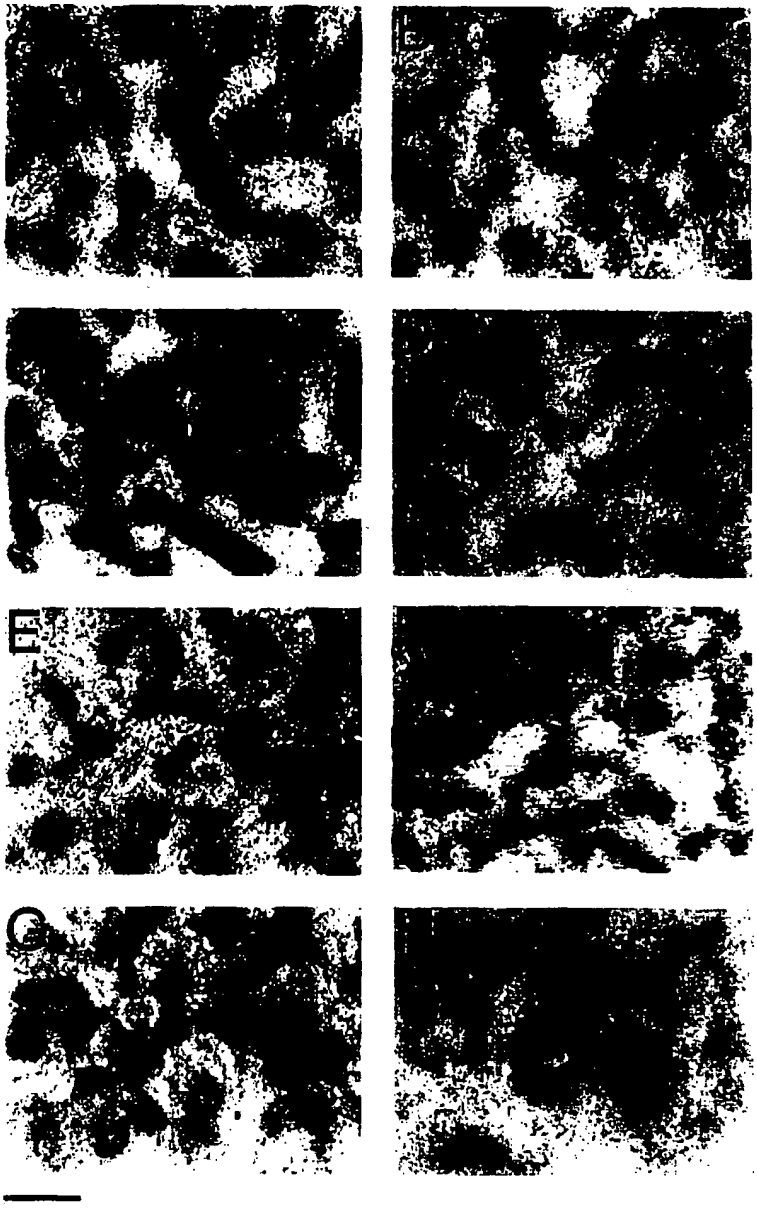


FIGURE 2A

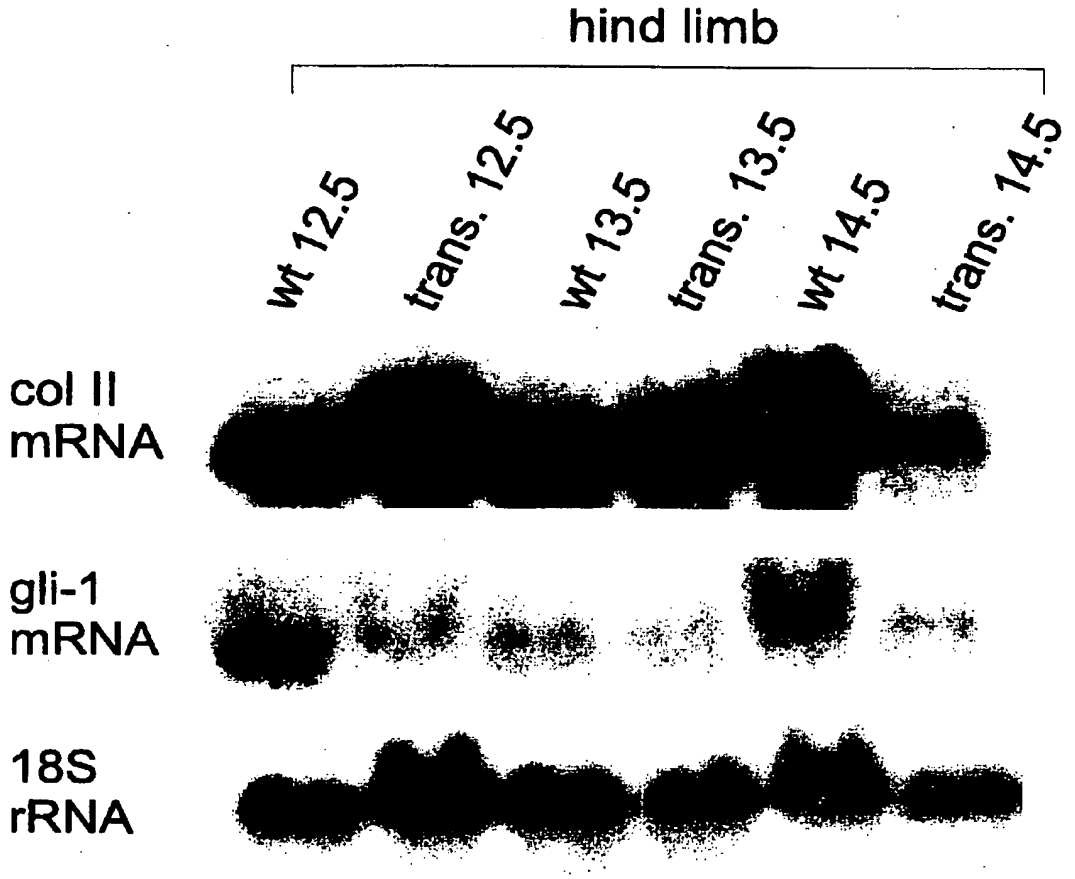

hind limb
wt 12.5
trans. 12.5
wt 13.5
trans. 13.5
wt 14.5
trans. 14.5
col II mRNA
gli-1 mRNA
18S rRNA

FIGURE 2B

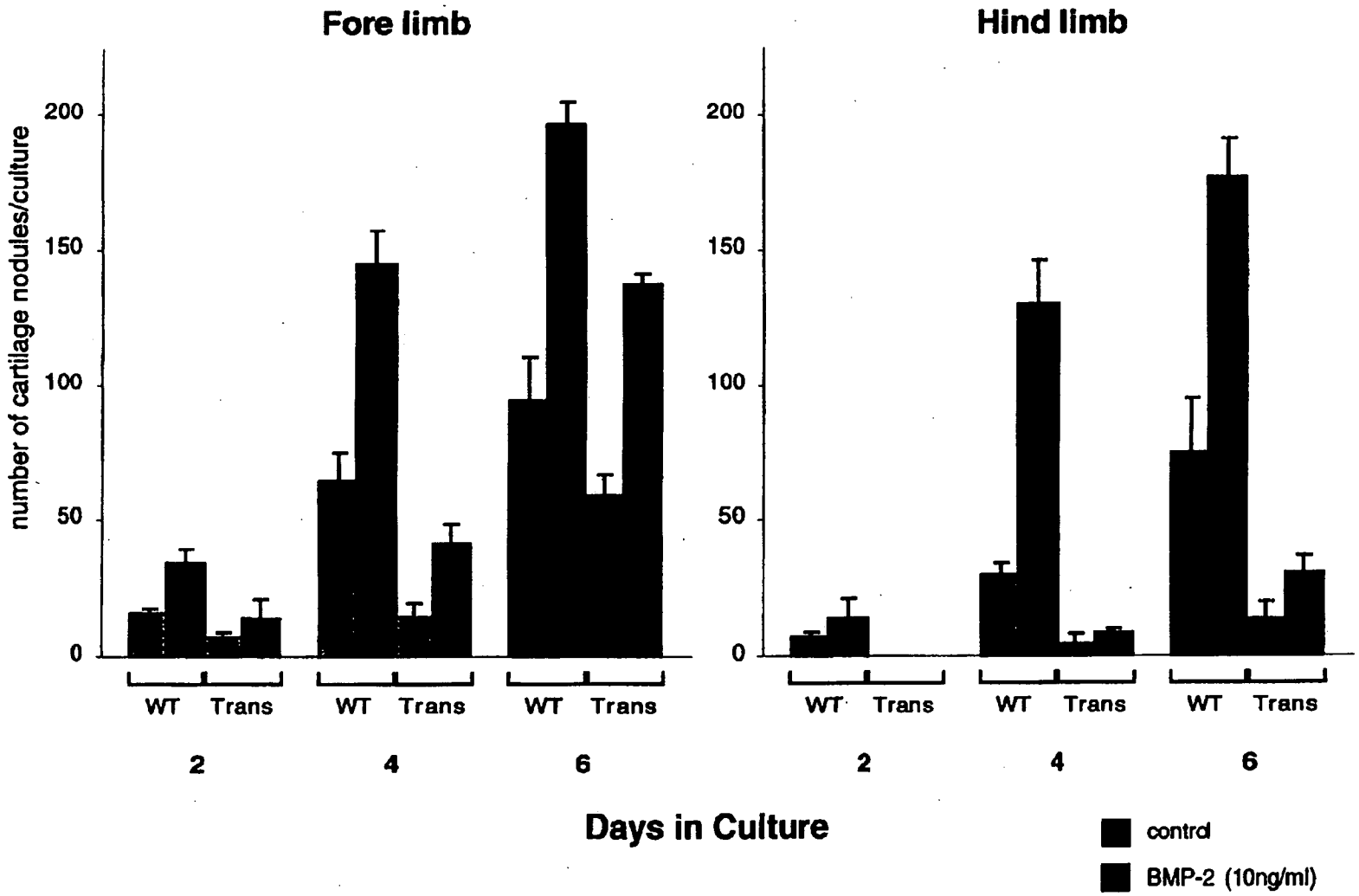

Fore limb
Hind limb
number of cartilage nodules/culture
200
150
100
50
0
WT
Trans
2
4
6
Days in Culture
control
BMP-2 (10ng/ml)

FIGURE 3A

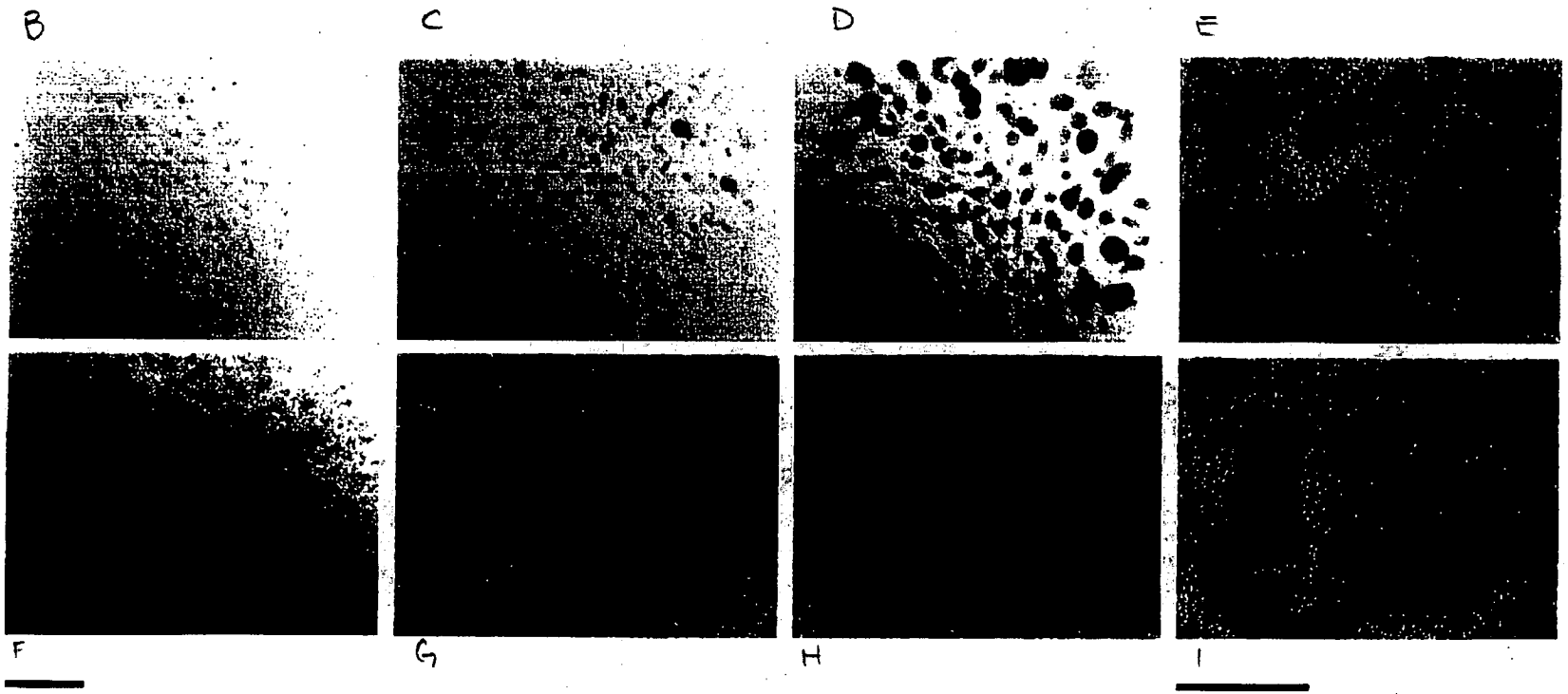


FIGURE 3B TO 3I

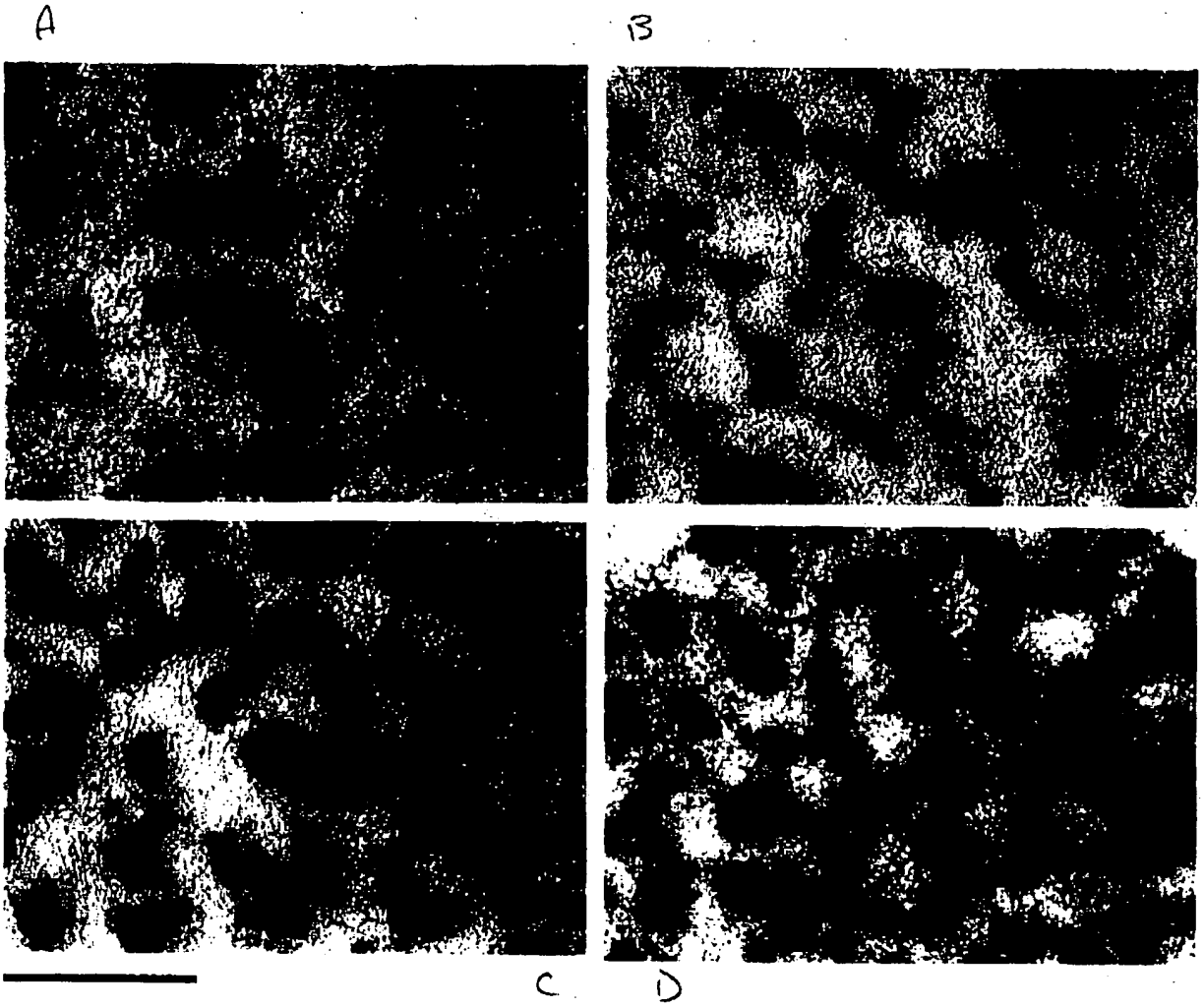
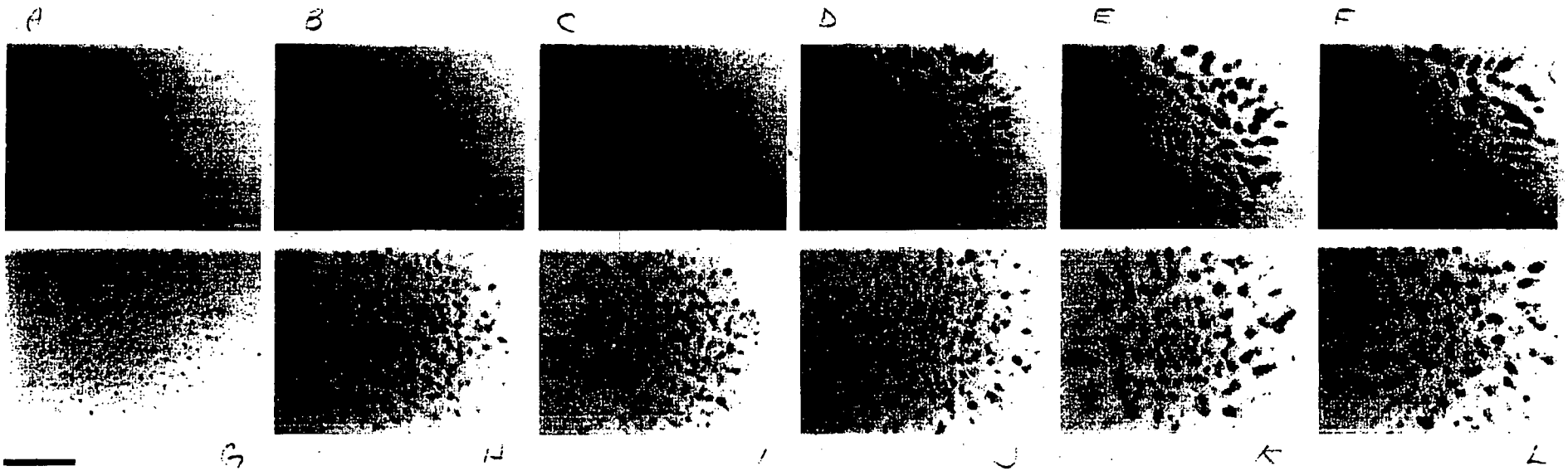


FIGURE 4



FIGURES 5A TO 5L

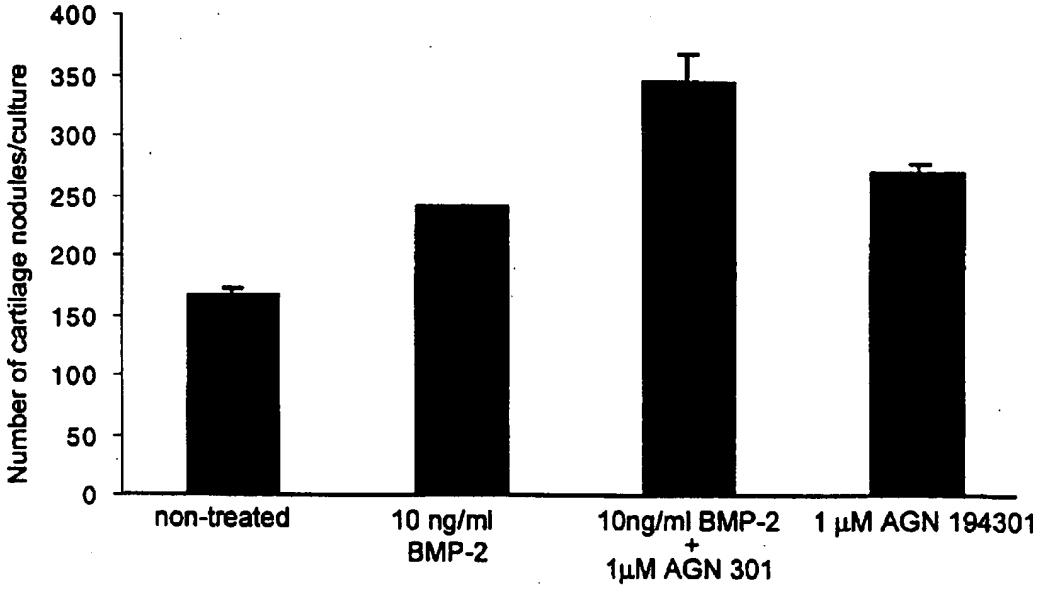
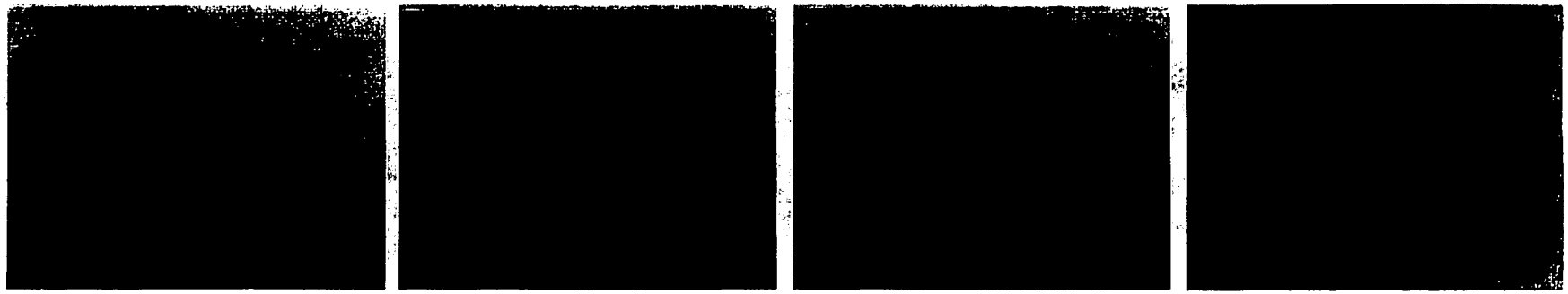

Number of cartilage nodules/culture
400
350
300
250
200
150
100
50
0
non-treated
10 ng/ml BMP-2
10ng/ml BMP-2 + 1µM AGN 301
1 µM AGN 194301

FIGURE 5M



FIGURES 6A TO 6D

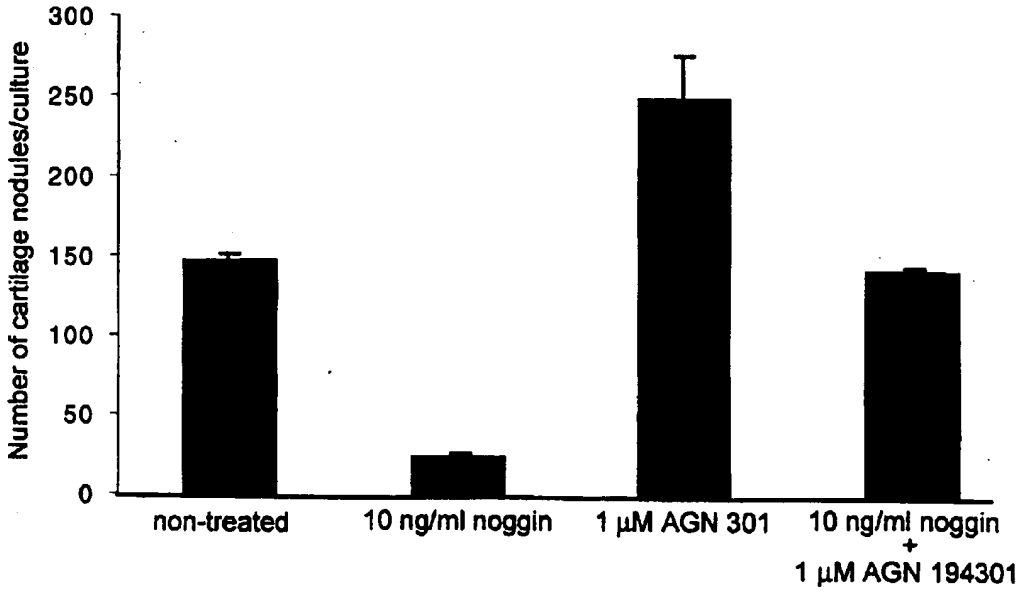

Number of cartilage nodules/culture
300
250
200
150
100
50
0
non-treated
10 ng/ml noggin
1 µM AGN 301
10 ng/ml noggin
+
1 µM AGN 194301

FIGURE 7